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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PO 9305 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION PLANT INDUSTRY filed on 19 September 1997.

I further certify that the name of the applicant has been amended to COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION and GRAINS RESEARCH AND DEVELOPMENT CORPORATION pursuant to the provisions of Section 104 of the Patent Act 1990.

I further certify that the annexed specification is not, as yet, open to public inspection.

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day of September 1998

KIM MARSHALL
<u>MANAGER EXAMINATION SUPPORT AND SALES</u>

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PROVISIONAL SPECIFICATION
for the invention entitled :

METHOD FOR ALTERING SEED COMPOSITION

The invention is described in the following statement :

METHOD FOR ALTERING SEED COMPOSITION

The present invention relates generally to a method for altering or modifying the composition of seeds of a plant.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Chimeric genes encoding sulfur-rich proteins have been transferred to plants for the purpose of increasing the sulfur amino acid content of the seed protein. For example, the transfer of a sunflower (*Helianthus annuus*) seed albumin (SSA) gene construct to *Lupinus angustifolius* in order to enhance the sulfur amino acid content and the nutritive value of the seed protein has been reported (Molvig et al., 1997). Plants such as canola (Altenbach et al., 1992), soybean (Townsend and Thomas, 1994) and narbon bean (Saalbach et al., 1995) have been transformed with genes encoding a methionine-rich protein from Brazil nut. In these cases, enrichment of seed protein with sulfur amino acids has been reported.

It has been found that transgenic soybeans containing the Brazil nut protein (BNP) at a level approximately equal to 4% of total seed protein had reduced levels of some endogenous sulfur-rich proteins, for example a storage protein, glycinin, and the Kunitz trypsin inhibitor (Townsend and Thomas, 1994). These effects on seed storage protein composition are similar to those which accompany sulfur stress in pea and lupin seeds. In the cases of peas and lupins, seeds grown under conditions of sulfur limitation contain decreased amounts of the storage proteins that contain sulfur amino acids, and increased amounts of storage proteins with little or no sulfur amino acids (Chandler et al., 1984,

Blagrove et al., 1976). In the case of the transgenic soybean, the methionine-rich BNP was made at the expense of endogenous sulfur-containing compounds.

Similarly, it has been reported that transgenic narbon beans expressing BNP do not contain increased levels of total sulfur in comparison to non-transgenic narbon beans (Muntz et al., 1997). This indicates that also in the narbon bean, a new sulfur sink causes re-routing of sulfur away from endogenous compounds. A significant part of the seed sulfur of narbon bean exists in the form of the dipeptide, γ-glutamyl cysteine (GEC). Preliminary results showed that GEC was reduced in transgenic narbon beans expressing BNP, and it has been proposed to exploit this strategy for reducing the content of the unpalatable GEC in narbon bean in order to increase its utilisation in animal feeds (Muntz et al., 1997).

In work leading to the present invention, the inventors have investigated a method for altering the composition of plant seeds by transferring to the genome of the plant a seed-specifically expressed gene encoding a protein rich in methionine and/or cysteine, such as sunflower seed albumin (SSA) containing 16% methionine and 8% cysteine (previously referred to as SFA8, Kortt et al., 1991).

The changes in seed composition that result from this process include an increase in the total protein content of the seeds (this has been achieved in pea, *Pisum sativum* and chickpea, *Cicer arietinum*), a decrease in seed fibre content (this has been achieved in narrow leaf lupin, *Lupinus angustifolius*) and a decrease in the content of endogenous anti-nutritional factors (this has been achieved in *Pisum sativum* and *Cicer arietinum*). More than one of these changes may occur in any given plant species, and these changes may occur in addition to or instead of an expected increase in the sulfur amino acid content of seed protein.

Accordingly, in one aspect of the present invention, there is provided a method of modifying the composition of seeds of a plant, said method comprising inserting into the genome of said plant a chimeric gene comprising a genetic sequence encoding a protein rich in methionine and/or cysteine under regulatory control of a promoter which is strongly

expressed in developing seeds of the plant.

The present invention also extends to a plant which has inserted into its genome a chimeric gene as broadly described above, as well as to seeds of such a plant.

As used herein, the terms "modifying" and "altering" shall be taken to refer to any alteration of seed composition and to include, but not be limited to, one or more of: (i) increase in the total protein content of seeds; (ii) reduction in the fibre content of seeds; (iii) alteration of the fibric quality of seeds; and (iv) reduction in the content of endogenous anti-nutritional factors in seeds. As previously discussed, such modifications and alterations may take place in addition to or instead of an increase in the sulfur amino acids content of the protein of seeds. As used herein, the term "sulfur amino acids" shall be taken to refer to methionine and/or cysteine in total.

The term "seeds" as used herein shall be taken to include both seeds and other storage organs of plants, such as tubers, specialised stems and the like.

The genetic sequence encoding a protein rich in methionine and/or cysteine is preferably the gene encoding sunflower seed albumin (SSA), however, the present invention is not restricted to the use of this particular preferred gene and extends to include a coding region specifying a methionine rich protein from Brazil nut (BNP) and any other coding region specifying a protein rich in methionine and/or cysteine, for example, a protein containing greater than 10% sulfur amino acids.

In a preferred embodiment, the present invention provides a method as broadly described above, wherein the chimeric gene comprises the SSA gene under regulatory control of a promoter which is strongly expressed in developing seeds of the plant.

Similarly, the promoter which is strongly expressed in developing seeds is preferably a pea vicilin gene promoter, however, the present invention is not restricted to the use of this

particular promoter and extends to include a promoter of a phaseolin gene from beans, and a promoter of a phytohemagglutinin gene from beans, as well as any other promoter which is strongly or specifically expressed in developing seeds. In the case where a plant is a monocotyledon, suitable promoters include, for example, a promoter of a high moleculan weight glutenin gene from wheat.

In another preferred embodiment, the present invention provides a method as broadly described above wherein the chimeric gene comprises a genetic sequence encoding a protein rich in methionine and/or cysteine under regulatory control of a pea vicilin gene promoter.

The plants to which the present invention may be applied include both dicotyledenous and monocotyledenous plants, including but not limited to, horticultural, vegetable, cereal or agricultural plants, such as pea, *Pisum sativum*, chickpea, *Cicer arietinum*, and narrow leaf lupin, *Lupinus angustifolius*, as well as monocotyledons such as maize, wheat, barley, rice, oats and sorghum.

A particularly preferred embodiment of the present invention is a method of modifying the composition of seeds of a plant by inserting into the genome of the plant a chimeric gene comprising a pea vicilin gene promoter, a coding region specifying sunflower seed albumin (SSA) and a terminator region from a pea vicilin gene.

Other preferred embodiments of this invention include:

(i) a method of increasing the protein content of seeds of *Cicer arietinum* or *Pisum sativum* by inserting into the genome of the plant a chimeric gene comprising a promoter strongly expressed in developing seeds and a coding region specifying a protein rich in methionine and/or cysteine, particularly a chimeric gene comprising a pea vicilin gene promoter, a coding region specifying sunflower seed albumin (SSA) and a vicilin gene terminator region;

(ii) a method of decreasing the fibre content (and/or altering fibre quality) of seeds of *Lupinus angustifolius* by inserting into the genome of the plant a chimeric gene

comprising a promoter strongly expressed in developing seeds and a coding region specifying a protein rich in methionine and/or cysteine, particularly a chimeric gene comprising a pea vicilin gene promoter, a coding region specifying sunflower seed albumin (SSA) and a vicilin gene terminator region;

(iii) a method of decreasing the content of anti-nutritional factors in seeds of *Lupinus angustifolius, Cicer arietinum,* or *Pisum sativum* by inserting into the genome of the plant a chimeric gene comprising a promoter strongly expressed in developing seeds and a coding region specifying a protein rich in methionine and/or cysteine, particularly a chimeric gene comprising a pea vicilin gene promoter, a coding region specifying sunflower seed albumin (SSA) and a vicilin gene terminator region.

Placing a genetic sequence which encodes a protein rich in methionine and/or cysteine under the regulatory control of a promoter means positioning the said genetic sequence such that expression is controlled by the promoter sequence. Promoters are generally, but not necessarily, positioned 5' (upstream) to the genetic sequences that they control. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function.

The gene construct for transfer to plants may further incorporate a selectable marker, such as *npt*II, hygromycin-resistance gene, a phosphinothricin-resistance gene or ampicillin-resistance gene, amongst others, associated with the transforming DNA to assist in cell selection and breeding. In addition, the gene construct may also incorporate a screenable marker, such as the *uid* A gene encoding the β-glucuronidase (GUS) enzyme.

Optional additional regulatory sequences which modulate the expression of genetic sequences may be included in the chimeric gene, for example a terminator sequence placed 3' or downstream of the genetic sequence encoding a protein rich in methionine and/or

cysteine. The term "terminator" as used herein refers to a DNA sequence at the end of a transcriptional unit encoding said protein, wherein said terminator signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. Examples of terminators particularly suitable for use in the chimeric genes of the present invention include a vicilin gene terminator, the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the *Oryza sativa* ADP glucose pyrophorylase gene terminator (t3'bt2), the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays* or the Rubisco small subunit (SSU) gene terminator sequences, amongst others.

The chimeric gene may be introduced into plant tissue, thereby producing a "transgenic plant", by various techniques known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques. Means for introducing chimeric genes into plant tissue include, but are not limited to, direct DNA uptake into protoplasts, PEG-mediated uptake to protoplasts, microparticle bombardment electroporation, microinjection of DNA, microparticle bombardment of tissue explants or cells, or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue. Methods for the *Agrobacterium*-mediated transformation of plant tissue will be well-known to those skilled in the art. A whole plant may be regenerated from the transformed plant tissue by methods which are also well-known to those skilled in the art.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a chimeric gene in accordance with the present invention. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The term

"organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers. The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Plant seeds and other storage organs are the major sources of energy and protein in animal diets. Protein is the higher value constituent. Diets for animals are formulated from a mixture of ingredients to contain optimal levels of protein and energy. Seeds containing significantly increased protein would be preferred ingredients in feed mixes containing other seeds rich in starch, for example unmodified cereals. Fibre, including non-starch polysaccharide (NSP) in lupins, is not readily utilised by non-ruminant animals, thus a reduction in seed fibre would increase the nutritive value of the seed for non-ruminant animals. In particular, the soluble fraction of NSP (including oligosaccharides) in lupins is anti-nutritional for pigs and poultry. A reduction in this fraction of lupin seeds (as has been achieved in transgenic *L. angustifolius* in accordance with this invention) would increase the nutritive value of lupins for non-ruminants. This modification could increase the market share of domestic lupins for feed formulations that currently use imported soybean meal. In particular, the modified lupins, peas and possibly chickpeas of the present invention may have greater applicability than their parental counterparts as protein sources in aquaculture feeds. Plant protein must be concentrated for use in aquaculture feeds because prawns and fish cannot metabolise plant fibre. High protein grains would be preferred base materials for the preparation of these concentrates. Furthermore, prawns have a high nutritional requirement for arginine. As well as having increased total protein, transgenic peas and chickpeas expressing SSA have been enriched in arginine compared to wild type, thus the modified seeds should have even higher nutritive value for prawns than for other animals. Nutritional supplements are particularly difficult to apply in aquaculture because of the problems associated with their delivery in a liquid medium.

Pulses are a major source of human food in many countries. Increasing the protein content, and/or decreasing the content of anti-nutritional factors of seeds including pea and

chickpea would increase their food quality for humans.

Protein content is a major determinant of quality and value in cereals. High protein cereals, for example wheat, would have increased value in many applications. High protein barley, maize, oats and sorghum should attract a price premium as improved ingredients in animal feeds

The protein of legume seeds is naturally low in the nutritionally essential amino acids, methionine and cysteine. The present inventors have transferred a chimeric gene encoding SSA (Fig. 1) to lupin, pea and chickpea with the original aim of increasing the methionine and cysteine content of the seed protein. The gene was incorporated into a DNA construct containing a *bar* gene as a selectable marker and a *uidA* gene as a screenable marker (Fig. 1). The construct was transferred to plants using *Agrobacterium tumefaciens* (eg. Molvig et al., 1997, the contents of which are incorporated herein by reference).

Further features of the present invention are more fully described in the accompanying Figure and in the Examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

In the Figures:

Figure 1 is a diagrammatic representation of a chimeric gene encoding SSA which has been transferred to lupins, peas and chickpeas.

EXAMPLE 1

Transgenic lupins containing SSA

Transgenic lupin seeds which contained SSA and which had elevated methionine content and enhanced nutritive value for rats have been produced by *Agrobacterium*-mediated

transformation using the chimeric gene of Figure 1 (Molvig et al., 1997). In addition to the expected increase in sulfur amino acid content of seed protein, it has been found that transgenic lupin seeds containing SSA have lower fibre content than seeds of the parental lupin variety grown under essentially identical conditions (Table 1). The change in fibre content was unexpected and unpredictable.

Table 1 : Fibre content of wild type lupins and transgenic lupins containing SSA

	[1]wild type control	[1]SSA transgenic, line 55-38
[2]Soluble NSP	14.4	10.9
Insol. NSP	27.3	26
[3]Total NSP	41.7	36.9
Lignin	0.9	1.1
[4]Total dietary fibre	42.6	38

All values are % dry matter

[1]The wild type control seed and the transgenic seed (line 55-38, which is the transgenic line described in Molvig et al., 1997) were grown in screen houses under the same conditions.

[2]NSP: Non-starch polysaccharide

[3]Total NSP is the sum of soluble and insoluble NSP

[4]Total dietary fibre is the sum of total NSP and lignin

EXAMPLE 2

Transgenic peas containing SSA

The SSA gene was transferred to peas by *Agrobacterium*-mediated transformation using the chimeric gene of Figure 1, and instead of having significantly increased proportions of sulfur amino acids, the transgenic seeds had approximately 30% (up to 50%) more total seed protein than seeds of the parental, wild type plants grown under the same conditions in the

glasshouse. As well as having increased protein, the transgenic seeds also had reduced starch content relative to control seeds. Table 2 shows that pea seeds of several transgenic lines, derived from two different cultivars (the garden pea, cultivar Greenfeast and the field pea, cultivar Laura) contained more nitrogen and less starch than controls. Seed nitrogen is mostly in the form of protein, therefore, it can be assumed that increased seed nitrogen equates to increased seed protein. This assumption was tested directly by using precipitation with tri-chloroacetic acid (TCA) to measure the protein content of glasshouse-grown wild type and transgenic peas (Table 3). The results showed that the increase in seed nitrogen reflected an increase in seed protein.

Table 2 : Composition of wild type peas and transgenic peas containing SSA

Pea line	[1]**seed nitrogen (% DM)**	**seed starch (% DM)**
Transgenic lines from cv Greenfeast		
115-14	6.26	
	6.13	30
136-27	5.7	
	5.5	34
133-87	4.61	
	5.26	33
133-48	5.28	
	5.71	31
133-54	4.3	
	6.22	31
133-97	4.83	
	5.14	29
133-77	5.26	
	4.77	33
133-30	4.9	
	5.14	30
mean	**5.3**	**31.4**
Greenfeast control	4.05	35
	4.34	36

mean	**4.2**	**35.5**
change (tg - control)	27%	- 12%
Transgenic lines from cv Laura		
816-77	4.78	
	4.45	47
809-100	5.55	
	4.8	48
800-27	5.14	
	5.8	46
mean	**5.09**	**47**
Laura control	3.3	54
	3.36	53
mean	**3.33**	**53.5**
change (tg - control)	53%	- 12%

[1]Determinations done in duplicate

Table 3 : Nitrogen content of wild type peas and transgenic peas containing SSA.

	Total seed nitrogen (% DM)	**TCA-insoluble seed nitrogen (% DM)**
Laura control	3.95	3.30
SSA transgenic	4.99	4.33
% change (tg-control)	26.3	31.3

Western blotting was used to confirm the presence of the sulfur-rich SSA in the transgenic pea seeds at a level equal to approximately 2% of total seed protein. X-ray fluorescence spectrometer analysis revealed that the organic sulfur content of transgenic pea seeds was increased by 21.1%. This increase was of similar magnitude to the increase in total seed nitrogen (26.3%, Table 3), therefore the seed protein was not enriched with respect to the sulfur-containing amino acids, methionine and cysteine. Determination of the amino acid

composition of wild type pea seeds and transgenic pea seeds containing SSA confirmed that the amounts of all the amino acids had increased in seed of the transgenic line (Table 4). The levels of most amino acids, including methionine and cysteine had increased to similar extents. The increases in valine and isoleucine were less than the average change (which was about 30%) and the increase in arginine was greater than the average change, therefore the transgenic seeds were somewhat lower in their proportions of valine and isoleucine and higher in their proportion of arginine than their wild type counterparts.

Table 4 : Amino acid content of wild type pea seeds and transgenic pea seeds containing SSA

Amino acid	**[1]Wild type pea**	**[1]Transgenic pea**	**% increase in transgenic**
aspartic acid	21.3	28.6	34
threonine	7.9	10.55	34
serine	9.45	13.21	40
glutamic acid	34.6	45.59	32
proline	7.97	10.12	27
glycine	9.0	11.28	25
alanine	9.07	11.55	27
valine	9.94	11.22	13
isoleucine	9.43	10.42	10
leucine	14.13	17.8	21
lysine	14.94	19.04	27
arginine	13.27	30.96	133
cysteine	**2.57**	**3.57**	**39**
methionine	**2.13**	**2.81**	**32**

[1]All figures are mg/g of seed sample

In addition to an increase in total seed protein, transgenic pea seeds containing SSA had reduced levels of the endogenous, sulfur-rich, anti-nutritional proteins, trypsin inhibitor and chymotrypsin inhibitor. The levels of these inhibitors were measured by quantifying the inhibitory effects of total protein extracts from wild type or transgenic peas on the activity of trypsin or chymotrypsin in *in vitro* assays (Tables 5 & 6). Transgenic peas had 65% of

the level of trypsin inhibitor in wild type seeds and 37% of the level of chymotrypsin inhibitor in wild type seeds.

Table 5 : Trypsin inhibitor content of wild type pea seeds and transgenic pea seeds containing SSA

Sample	**Trypsin activity (TAME units/min)**	**% Inhibition**	**inhibition by transgenic as % of inhibition by wild type**
trypsin alone	70.08		
trypsin + 4 μg WT[1]	56.41	20.33	
trypsin + 4 μg Tg[2]	61.42	13.24	65.13

[1]4 μg of total protein extracted from wild type pea seed flour

[2]4 μg of total protein extracted from transgenic pea seed flour

Table 6 : Chymotrypsin inhibitor content of wild type pea seeds and transgenic pea seeds containing SSA

Sample	**Chymotrypsin activity (OD units/min)**	**% Inhibition**	**inhibition by transgenic as % of inhibition by wild type**
chymotrypsin alone	12.17		
chymotrypsin + 20 μg WT[1]	9.82	19.28	
chymotrypsin + 20 μg Tg[2]	11.3	7.12	37

[1]20 μg of total protein extracted from wild type pea seed flour

[2]20 μg of total protein extracted from transgenic pea seed flour

EXAMPLE 3

Transgenic chickpeas containing SSA

The chimeric SSA gene was transferred to desi type chickpeas, cultivar Semsen, by *Agrobacterim*-mediated transformation using the chimeric gene of Figure 1, and produced transgenic seeds with approximately 30% more total protein (TCA-insoluble nitrogen) than seeds of the parental, wild type plants grown under the same conditions in the glasshouse (Table 7). As well as having increased protein, the transgenic seeds had reduced starch content relative to control seeds.

Table 7 : Nitrogen content of wild type chickpeas and transgenic chick peas containing SSA.

	Total seed nitrogen (% DM)	**TCA-insoluble seed nitrogen (% DM)**
Semsen control	2.88	2.3
SSA transgenic	3.91	3.0
% change	35.8	31.8

Transgenic chickpea seeds contained SSA at a level equal to approximately 5% of total seed protein. X-ray fluorescence spectrometer analysis revealed that the organic sulfur content of transgenic chickpea seeds was increased by 30.8%. This increase was of similar magnitude to the increase in total seed nitrogen (35.8%, Table 7), therefore the seed protein was not enriched with respect to the sulfur-containing amino acids, methionine and cysteine. Determination of the amino acid composition of wild type chickpea seeds and transgenic chickpea seeds containing SSA confirmed that the amounts of all the amino acids had increased in seed of the transgenic line (Table 8). The levels of most amino acids had increased to similar extents (about 25%). The increase in cysteine was less than the average

change and the increase in methionine was greater than the average change, but the change in the two combined (30% for cys + met) was about the same as the average change (25%). Thus, although the transgenic chickpea seeds did contain more sulfur amino acids than control seeds, they did not have higher proportions of total sulfur amino acids than control seeds. This was similar to what had been observed in transgenic peas. Transgenic chickpea seeds were also somewhat richer in arginine than control chickpea seeds.

Table 8 : Amino acid content of wild type chickpea seeds and transgenic chick pea seeds containing SSA

Amino acid	**[1]Wild type chick pea**	**[1]Transgenic chick pea**	**% increase in transgenic**
aspartic acid	19.3	24.2	25
threonine	6.9	8.0	16
serine	9.7	12.4	28
glutamic acid	28.2	36.4	29
proline	8.2	9.7	18
glycine	7.0	8.5	21
alanine	7.4	9.1	23
valine	8.3	1.03	24
isoleucine	7.9	9.9	25
leucine	13.4	16.8	25
tyrosine	5.4	6.5	20
phenylalanine	1.01	1.31	30
lysine	12.1	14.7	21
arginine	15.3	23.0	50
cysteine	**3.4**	**3.7**	**9**
methionine	**3.0**	**4.6**	**53**
cys + met	**6.4**	**8.3**	**30**

[1]All figures are mg/g of seed sample

In addition to an increase in total seed protein, chickpea seeds containing SSA had reduced levels of the endogenous, sulfur-rich, anti-nutritional proteins, trypsin inhibitor and chymotrypsin inhibitor. The levels of these inhibitors were measured by quantifying the

inhibitory effects of total protein extracts from wild type or transgenic chickpeas on the activity of trypsin or chymotrypsin in *in vitro* assays (Tables 9 & 10). Transgenic chickpeas had 49% of the level of trypsin inhibitor in wild type seeds and 44% of the level of chymotrypsin inhibitor in wild type seeds.

Table 9 : Trypsin inhibitor content of wild type chickpea seeds and transgenic chickpea seeds containing SSA

Sample	**Trypsin activity (TAME units/min)**	**% Inhibition**	**inhibition by transgenic as % of inhibition by wild type**
trypsin alone	207		
trypsin + 6 μg WT[1]	98.5	52.4	
trypsin + 6 μg Tg[2]	153.8	25.7	49

[1]6 μg of total protein extracted from wild type chickpea seed flour

[2]6 μg of total protein extracted from transgenic chickpea seed flour

Table 10 : Chymotrypsin inhibitor content of wild type chickpea seeds and transgenic chickpea seeds containing SSA

Sample	**Chymotrypsin activity (OD units/min)**	**% Inhibition**	**inhibition by transgenic as % of inhibition by wild type**
chymotrypsin alone	14.94		
chymotrypsin + 12 μg WT[1]	9.91	33.7	
chymotrypsin + 12 μg Tg[2]	12.72	14.86	44.1

[1]12 μg of total protein extracted from wild type chickpea seed flour

[2]12 μg of total protein extracted from transgenic chickpea seed flour

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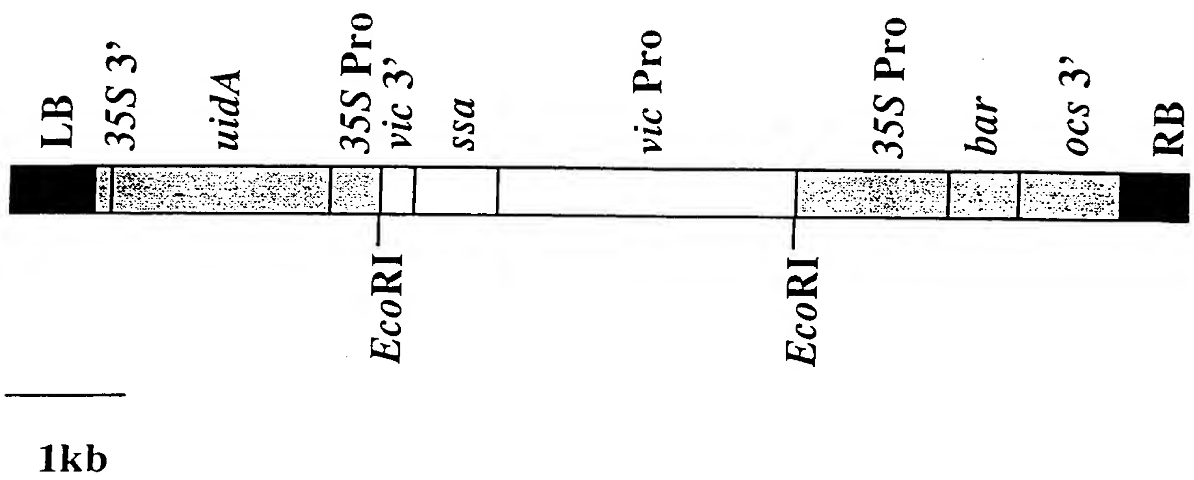
Figure 1



The multi-gene construct transferred to lupins, peas and chick peas.

The construct contains three chimeric genes; *35S-uidA*, encoding the reporter enzyme b-glucuronidase or GUS; *vic-ssa*, encoding the sunflower seed albumin; and *35S-bar* encoding the selectable marker phosphinothricin acetyltransferase, or PAT.
LB and RB, left and right T-DNA borders from *A. tumefaciens*; *35S* 3', 3' flanking region from the *35S* gene of cauliflower mosaic virus(CaMV); *uidA*, protein-coding region from the *uidA* gene of *E. coli*; *35S* Pro, promoter from the *35S* gene of CaMV; *vic* 3', 3' flanking region from a vicilin gene from pea; *ssa*, protein-coding region from the *ssa* gene from sunflower (*Helianthus annuus*); *vic* Pro, promoter from a pea vicilin gene ; *bar*, protein-coding region from the *bar* gene of *Streptomyces hygroscopicus*; *ocs* 3', 3' flanking region from the octopine synthase gene of *A. tumefaciens*. Details of the gene constructions are published in Molvig et al., and references therein.